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The catecholic antioxidant piceatannol is an effective nitrosation inhibitor via an unusual double bond nitration

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Abstract—Piceatannol (**1**) was found to be more effective than caffeic acid, an established antinitrosating agent, in inhibiting N-nitrosation of 2,3-diaminonaphthalene. Product analysis of the reaction mixture of **1** (20 μM) with nitrite ions (80 μM) at pH 3.0 and at 37 °C showed conversion to a single major nitration product, (*E*)-3,3′,4,5′-tetrahydroxy-β-nitrostilbene (**2**) (68% yield). This would result from an unexpected nitration at the double bond sector via the 4-phenoxyl radical, which was analyzed at the unrestricted DFT level.

The study of the reactions of polyphenolic constituents of the diet with mutagenic nitrosating species is an area of great promise in cancer preventive strategies aimed at controlling tumour initiating events. Several catechol compounds of plant origin have emerged as efficient scavengers of nitrosating species and have been suggested to play a role in lowering the impact of nitrosation reactions on DNA base deamination and carcinogenic *N*-nitrosamine formation following elevated nitrite intake.[1,2]

So far, most of the interest in this field has been focused on caffeic acid and its esters, which rank among the most efficient nitrosation inhibitors.[3–5] Hydroxytyrosol and related phenols from extra-virgin olive oil,[6] and green tea catechins[7–9] have also been shown to act as effective inhibitors of nitrosation processes.

An attractive candidate, in the quest for novel efficient antinitrosating agents, is piceatannol (*trans*-3,3′,4,5′-tetrahydroxystilbene, **1**), a catechol component of red wine polyphenolics[10] found also in *Vaccinium* berries.[11] Studies in the chemistry and bioactivity of **1** have underscored its potent antioxidant, anti-inflammatory, anti-proliferative and cancer chemopreventive activities,[12–17] but little is known about its potential nitrite scavenging and antinitrosaminic properties.

In the present paper, we provide the first evidence for the effective inhibitory properties of **1** in a model system of N-nitrosation processes, involving the reaction of 2,3-diaminonaphthalene (DAN) with nitrous acid leading to fluorescent naphtho[2,3-*d*]triazole formation.[3] For comparative purposes, 3,4-dihydroxycinnamic acid (caffeic acid), an established inhibitor of DAN nitrosation,[3] was also investigated.[†]

The results in Figure 1 show that, under the typical conditions of the assay, **1** was more effective than caffeic acid in inhibiting fluorophore development over the whole concentration range examined. From fluorescence measurements, the ratio of the kinetic constants $k_1/k_{\text{caffeic acid}}$ for the reactions of **1** and caffeic acid with nitrite could be calculated as 1.7 ± 0.5 using the equation below:[3]

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[†] Compound **1** or caffeic acid was incubated at 0–1 mM concentration in 50 mM acetate buffer (pH 4.0, 200 μL) in the presence of DAN (0.2 mM) and sodium nitrite (20 mM). After 30 min 50 mM phosphate buffer (pH 7.4, 1.8 mL) was added to stop the reaction. Naphtho[2,3-*d*]triazole was quantified by measuring the fluorescence of each sample using an excitation wavelength of 375 nm and an emission wavelength of 450 nm.

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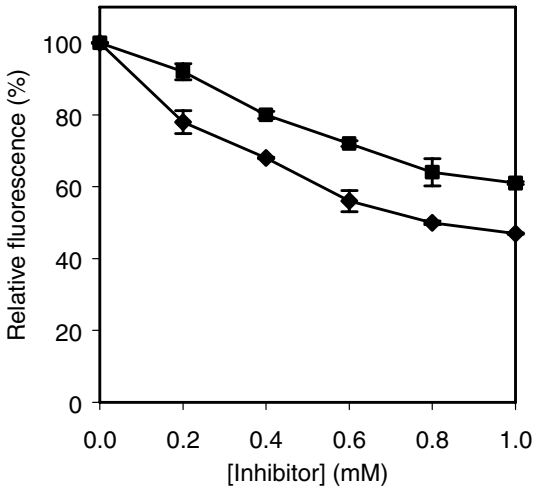


Figure 1. Inhibition of N-nitrosation of DAN by 1 (♦) and caffeic acid (■) measured as fluorescence emission at 450 nm of naphtho[2,3-*d*]triazole. Relative fluorescence represents the ratio of fluorescence values measured in the presence versus that determined in the absence of the inhibitor. Shown are the mean ± SD values for two separate experiments.

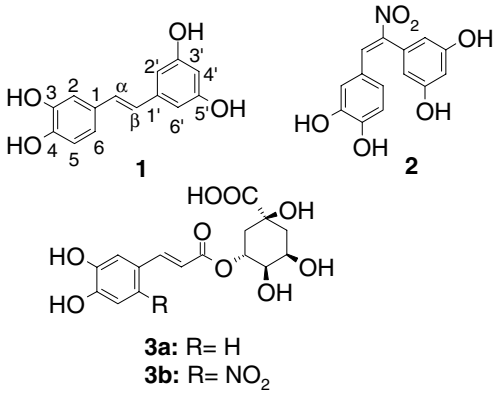
$$f/F = 1 - k_{\mathrm{In}}[\mathrm{In}]/k_{\mathrm{DAN}}[\mathrm{DAN}],$$

where f and F are the fluorescence intensities determined in the presence and in the absence of the inhibitor (In), respectively. For the reaction of DAN with nitrite the reported rate constant of 8.6×10^9 M^{-1} s^{-1} was used.[3]

Based on this observation, the reaction of **1** with nitrite ions was then investigated under conditions mimicking at best those found in human gastric fluid during a regular intake of physiological levels of nitrite.

At 20 μM concentration, **1** reacted with sodium nitrite (4 M equivalents added in four portions at 30 min intervals of time) in 0.1 M phosphate buffer (pH 3.0) at 37 °C to give a major product, which was isolated in pure form in 68% yield from a preparative scale reaction by preparative HPLC[‡] of the ethyl acetate extractable fraction.

The product displayed absorption maxima at 256, 277 and 373 nm (CH_3OH), shifting to 285 and 472 nm in CH_3OH/0.1 M $NaHCO_3$ solution (pH 8). It gave pseudomolecular ion peaks at *m/z* 312 $[M+Na]^+$ and at *m/z* 288 $[M-H]^-$ in the ESI(+) and ESI(−)/MS spectrum, respectively, suggesting a nitrated derivative. The ^1H NMR spectrum showed a set of aromatic/olefinic proton resonances which lacked the expected 1H doublets for the *trans* H-α and H-β protons. Besides the resonances of the resorcin moiety, appearing as a doublet (2H, $J = 2.0$ Hz) at δ 6.25 and a triplet (1H, $J = 2.0$ Hz) at δ 6.44, showing one-bond correlation with two carbon resonances at δ 109.9 and 105.1, respectively, and those of the catechol ring (broad 3H singlet at δ 6.71 due to three overlapped proton resonances correlating with three carbon signals at δ 116.6, 119.5 and 126.5), a diagnostic feature was a down-field 1H singlet at δ 8.07 correlating with a carbon signal at δ 136.1. These data allowed unambiguous identification of the product as the unexpected (*E*)-3,3′,4,5′-tetrahydroxy-β-nitrostilbene (**2**).



This structural assignment was deduced from extensive 2D NMR analysis: in particular, the position of the nitro group was determined by distinct cross-peaks in the ^1H,^{13}C HMBC spectrum between the signal at δ 8.07 and the carbon resonances at δ 119.5 and 126.5 due to the catechol moiety. The *E* configuration at the double bond was inferred from the characteristic chemical shift of the H-α proton experiencing the magnetic anisotropy effect of the adjacent nitro group,[18] and was confirmed by a correlation in the ROESY spectrum between the resorcin doublet at δ 6.25 and the overlapped catechol signals at δ 6.71.

The geometrical features of **2** were examined by DFT optimizations,[19] using the hybrid PBE0 functional[20] in combination with a medium-size basis set of the Pople series, namely 6–31 + G(d,p).[21] Absolute NMR shielding tensors were computed within the Gauge-Including Atomic Orbitals (GIAO) ansatz[22] at the PBE0/6–311 + G(d,p) level, which has proven reliable in similar applications,[23] and were converted to isotropic chemical shifts using as reference the values obtained at the same level for benzene. Since the experimental NMR data have been collected in acetone, the polarizable continuum model (PCM)[24] was used throughout to simulate the solvent, in combination with the United Atom for Hartree-Fock (UAHF) parametrization for atomic radii.[25] A few asymmetric conformers that were energy minimized initially evolved to structures of C_s symmetry, with the resorcinol ring perpendicular to a plane containing the whole nitrostyrene moiety. Several additional optimizations were performed on symmetric conformers, differing by a 180° rotation of the catechol ring and/or in the orientation of the phenolic hydroxyl groups, and NMR calculations were carried out on the main minima (Fig. 2).

The computed chemical shifts were Boltzmann averaged and are compared to the experimental values in Table 1. The data indicate a satisfactory agreement, with a noteworthy simulation of the unexpectedly similar proton shifts of the catechol ring.

[‡] An octadecylsilane coated column (250 × 22 mm, 10 μm particle size) was used, at a 10 mL/min flow rate; eluent: 1% TFA/methanol = 70/30.

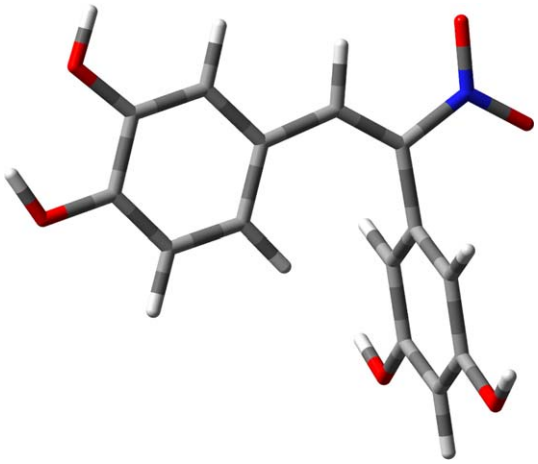


Figure 2. Energy minimized structure of **2** (C_s symmetry conformer).

Table 1. NMR spectral data of **2** (acetone-d_6)

	^{1}H (J, Hz)		^{13}C	
	Experimental	Calcd	Experimental	Calcd
1	—	—	125.0	122.7
2	6.71 (br s)	6.56	119.5	119.1
3	—	—	147.0	143.0
4	—	—	151.0	148.2
5	6.71 (br s)	6.53	116.6	113.9
6	6.71 (br s)	6.40	126.5	127.5
α	8.07 (s)	8.28	136.1	140.4
β	—	—	148.5	144.6
1′	—	—	134.2	135.5
2′/6′	6.25 (d, 2.0)	5.92	109.9	106.2
3′/5′	—	—	161.0	159.9
4′	6.44 (t, 2.0)	6.28	105.1	100.3

Careful HPLC analysis of the reaction mixture failed to show the presence of additional nitration products (UV evidence), the remainder of the mixture being made up of unidentified species probably derived from oxidative processes. Notably, when the reaction was run at pH 1.0, a marked decrease in the yield of **2** (less than 1%) was observed. This was shown to be due to the instability of **2**, which was converted to unidentified species. A greater stability of **2** was observed, however, at pH 3.0.

The observed site-specific nitration of **1** on the double bond was unexpected, since *o*-diphenols bearing a conjugated double bond, such as chlorogenic acid (**3a**) and other caffeic acid esters, usually react with acidic nitrite to give ring nitrated products, for example, 6-nitrochlorogenic acid (**3b**).[4,5]

To gain some mechanistic insights, in separate experiments it was found that the tetra-*O*-methyl derivative of **1** (prepared with methyl iodide following a reported procedure),[26] to which phenolic oxidation is precluded, does not react with acidic nitrite under the usual reaction conditions. This observation points to an oxidation step as a necessary requisite for nitration of **1**. This would be carried out by HNO_2 or NO_2 ($E_0 = 0.99$ V)[27] derived by decomposition of HNO_2 according to the following main equations:

$$NO_2^- + H^+ \rightleftarrows HNO_2$$

$$HNO_2 + H^+ \rightleftarrows NO^+ + H_2O$$

$$NO^+ + NO_2^- \rightleftarrows N_2O_3$$

$$N_2O_3 \rightleftarrows NO + NO_2$$

As a result, one-electron oxidation of **1** would lead to the corresponding semiquinone. This may disproportionate to give the *o*-quinone which may undergo nucleophilic attack by nitrite ions, as previously suggested in the case of caffeic acid derivatives, including **3a**.[4,5,28,29] This route, however, was ruled out in the light of separate experiments, in which the *o*-quinone of **1** was generated in situ under different conditions, that is, by tyrosinase-catalyzed or ferricyanide-promoted oxidation of **1** at pH 7,[30,31] in the presence of excess NO_2^- (5 M equivalents) without detectable conversion to **2**. Moreover, from inspection of the LUMO[32] of the putative *o*-quinone of **1** shown in Figure 3, the β-position did not appear as the most reactive electrophilic site with respect to the other conceivably reactive positions.

Accordingly, a free radical coupling mechanism for the nitrous acid-induced nitration of **1** was proposed, which is schematically illustrated in Figure 4.

In the proposed scheme, one-electron oxidation of **1** at the 4-OH group gives a highly delocalized radical (semiquinone), akin to that from resveratrol.[33] Semiquinone intermediates are known to be formed by oxidation of stilbene catechols and have been detected under neutral conditions in the presence of Mg^{2+} or Zn^{2+} as spin stabilizing agents.[34] Homolytic coupling of the semiquinone with NO_2 at the β-position would then give **2**.

The results of a DFT investigation of the 4-phenoxyl radical of **1** are consistent with those of the proposed free radical mechanism.[35] Figure 5 depicts the singly occupied molecular orbital (SOMO) of one conformer of the 4-phenoxyl radical of **1** in aqueous solution. The orbital is π in character, and has an appreciably

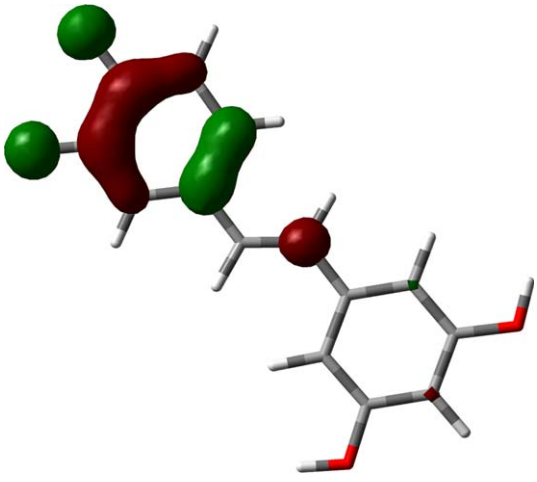


Figure 3. LUMO of the *o*-quinone of **1**.

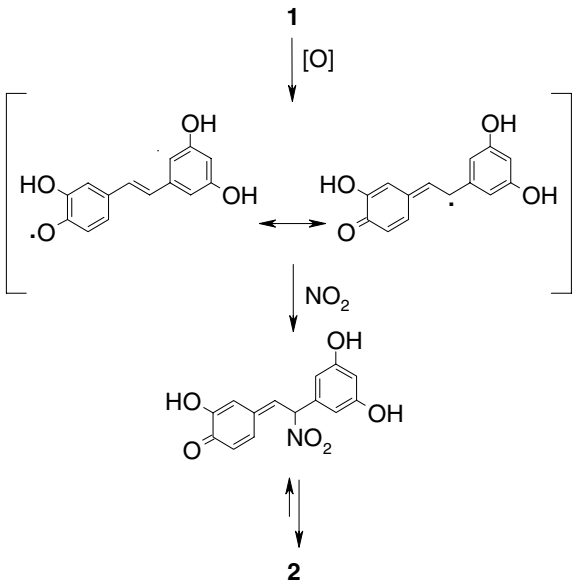


Figure 4. Proposed mechanism for the acid-promoted regioselective nitration of **1** with nitrite ions under biomimetic conditions.

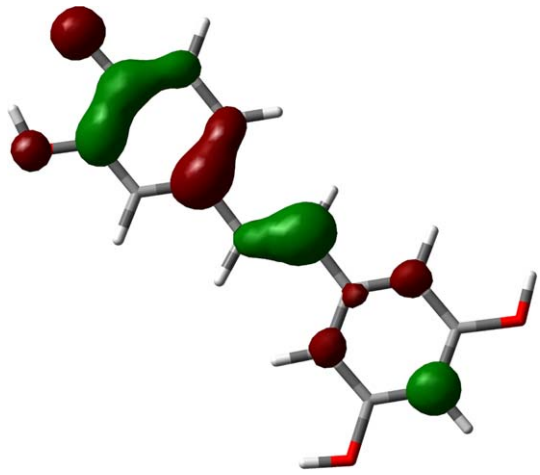


Figure 5. SOMO of the 4-phenoxyl radical of **1** (conformer #1).

larger coefficient on the β-position, that is, the one involved in nitration, than on the other conceivably reactive site, namely C-5. The overall picture is essentially unchanged for the other conformer of the radical, both in vacuo and in aqueous solution.

Table 2. Selected Mulliken atomic spin densities of the 4-phenoxyl radical of **1**

	In vacuo		Aqueous solution	
	Conf. #1	Conf. #2	Conf. #1	Conf. #2
Relative energy (kcal/mol)	0.0	0.0	0.0	0.2
C-β	0.297	0.332	0.300	0.333
C-5	0.194	0.239	0.163	0.204
C-2′	0.099	0.106	0.102	0.109
C-4′	0.110	0.119	0.118	0.127
C-6′	0.087	0.094	0.091	0.098

To allow for a more quantitative comparison, the Mulliken atomic spin densities on the relevant double bond and ring positions were determined. Data in Table 2 indicate that the spin density on C-β is much higher than on the C-5 ring position. Appreciable spin density is also present on the 2′, 4′, and 6′ positions of the resorcinol ring.

Despite the 3,4-dihydroxystyryl moiety in common with caffeic acid esters, for example, **3a**, only **1** reacts with acidic nitrite via coupling of the 4-phenoxyl radical with NO_2. This may reflect an additional stabilization of this radical by the resorcin ring, as shown by the significant SOMO coefficient at the 4′-position of **1** denoting extensive spin delocalization (Fig. 5) as well as by spin density data (Table 2). In the case of **3a** and other caffeic acid esters, the 4-phenoxyl radicals would expectedly be less stable, escaping trapping by NO_2. They would preferentially disproportionate to give the *o*-quinones,[4,5,29] which are amenable to nucleophilic attack by nitrite ions to give ring nitration products.

In conclusion, we have shown that **1** is an efficient inhibitor of N-nitrosation processes in vitro. It is more effective than caffeic acid, an established nitrosation inhibitor,[3–5] and acts through a redox pathway involving reaction of the 4-phenoxyl radical with NO_2 at the double bond sector. These results may disclose new mechanisms underlying the potent cancer chemopreventive activity of **1**.

Acknowledgments

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